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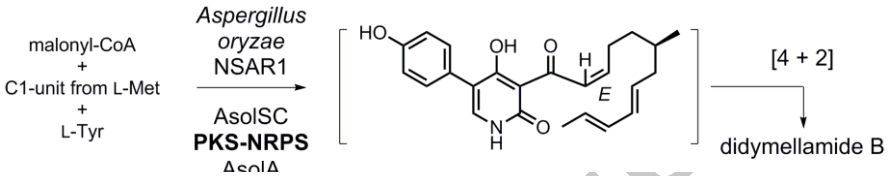

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Genome mining approach for harnessing the cryptic gene cluster in *Alternaria solani*: Production of PKS-NRPS hybrid metabolite, didymellamide B

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ABSTRACT

Using a heterologous expression system in *Aspergillus oryzae* we characterized cryptic biosynthetic genes found in *Alternaria solani*. This system enabled the isolation of an antifungal metabolite, didymellamide B, which was previously not detected in the extract of the *A. solani*. The co-production and chemical transformation of didymellamide congeners enabled us to decipher the biosynthetic pathway of didymellamide B as follows: 1) formation of a linear polyketide chain with 3-acyltetramate, 2) oxidative ring expansion to yield a 2-pyridone skeleton, and 3) non-enzymatic *endo*-selective [4+2] cycloaddition to afford a *trans*-decalin. These results demonstrate the robustness and reliability of the *A. oryzae* expression system.

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Introduction

A recent bioinformatics analysis of the various fungal genome has shown that 20–100 biosynthetic gene clusters of secondary metabolites are found in a single strain, and nearly half of these are polyketide synthase (PKS) genes, suggesting that polyketides are the most abundant metabolites in fungi.[1] Functions of these gene clusters have however not been examined. Genome mining using heterologous production are one of the most versatile methods used for deciphering cryptic gene clusters. Recently, we have successfully synthesized meroterpenoids, terpenes and polyketide metabolites using the *Aspergillus oryzae* heterologous expression system.[2] As part of our ongoing project on the total biosynthesis of bioactive fungal polyketides such as cholesterol-lowering lovastatin and antifungal agent griseofulvin, we focus on the heterologous expression of gene clusters, including the PKS or PKS-non-ribosomal peptide synthetase (PKS-NRPS).[3]

Alternaria solani is a phytopathogenic fungus, which causes potato early blight.[4] This fungus produces various types of phytotoxic metabolites, including polyketides alternariol, altersolanol A, altertoxin, macrosporin, and solanapyrone.[4] The biosynthesis of solanapyrone that involves an intriguing Diels-Alderase (DAase) Sol5 has been extensively studied.[5] Heterologous expression of several *A. solani* PKS successfully yielded polyketide pyrones.[6] The polyketide metabolite, alternaric acid was isolated as an antifungal agent in 1949 and was shown to contribute the disease development caused by *A. solani*.[7] Based on conventional feeding experiments with isotope labeled acetate, we previously proposed a unique strategy for the construction of the polyketide scaffold, including a condensation of two different polyketide chains.[8]

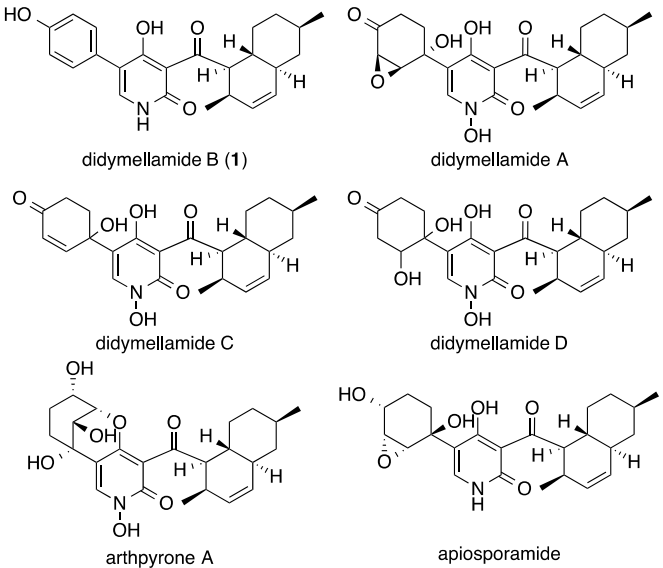

Figure 1. Fungal PKS-NRPs hybrids harboring 2-pyridone and decalin moieties.

During the biosynthetic study of alternaric acid, we faced an interesting phenomenon on a single gene cluster consisting of a functionally unknown PKS-NRPS. Although the relevant genes were apparently expressed, no corresponding metabolite was detected on several analytical methods. We became interested in this unusual phenomenon on the PKS and decided to determine the structure of the relevant polyketide. In this communication, we describe the successful production of didymellamide B (**1**) [9] polyketide metabolites with pyridone and decalin moieties by the heterologous expressions of three genes using *A. oryzae* host

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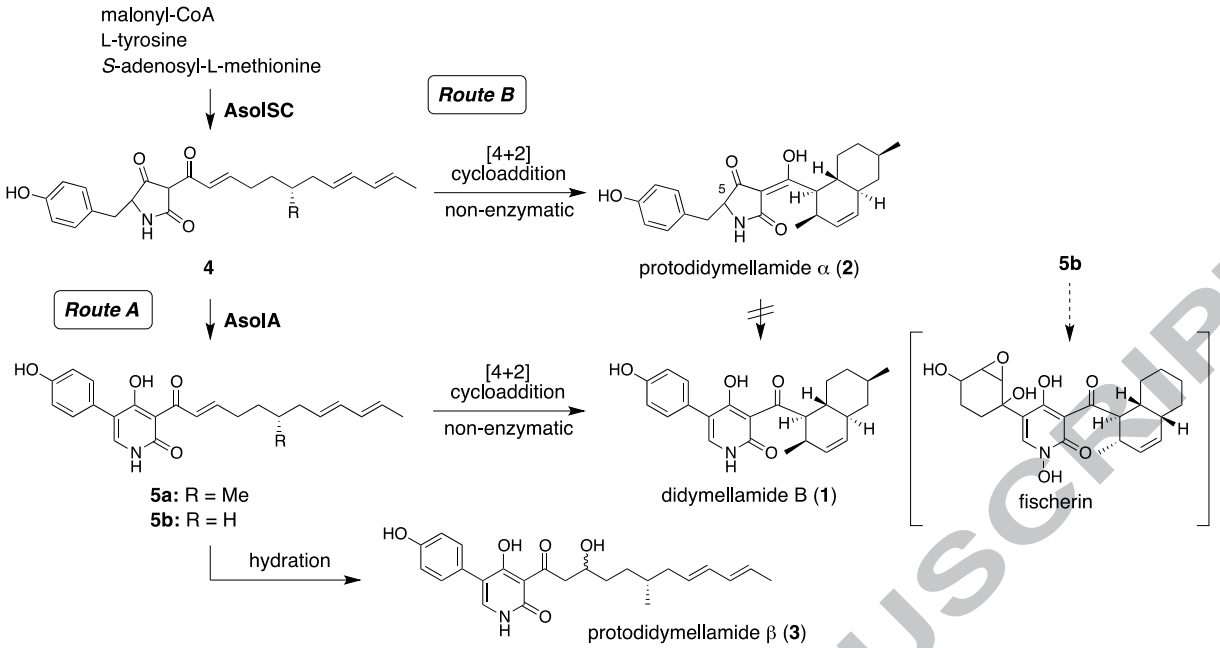

Scheme 1. Proposed biosynthetic pathway of didymellamide B.

(Figure 1). Co-production of didymellamide congeners enabled us to propose the biosynthetic pathway of **1** involving a non-enzymatic [4+2] cycloaddition.

Results and discussion

In the draft genome sequence of the alternaric acid producer *A. solani* A-17, we found 11 highly reducing PKS (HR-PKS) genes by a BLAST search. RT-PCR of those genes in the mycelia of *A. solani* under alternaric acid producing conditions showed apparent expression of the PKS-NRPS gene *asolS* (Figure S1). The gene cluster comprising the *asolS* gene contained the trans-acting ER (*asolC*), two cytochrome P450 genes (*asolA* and *asolB*) and other modification genes (*asolDEH*) encoding flavoprotein, methyltransferase, short-chain dehydrogenase, and auxiliary proteins (*asolFG*) (Figure S2). Three *asolSCA* showed high similarity (49-60% identity) to the biosynthetic genes of tenellin,[10] a PKS-NRPS product with a pyridone moiety. Next, we attempted to isolate the relevant polyketide metabolites in the extracts from several fermentation media but failed to detect any metabolite with TLC, HPLC and LC-MS, indicating its low productivity.

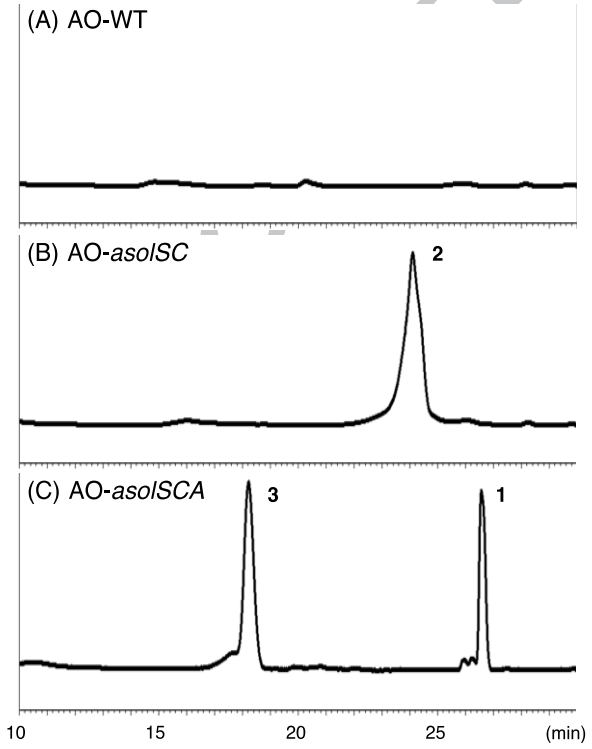

Figure 2. HPLC profiles of the metabolites from (A) wild type strain 283 nm, (B) transformant with *asolSC* 283 nm, and (C) transformant with *asolSCA* 347 nm.

To determine structure of the polyketide metabolite, we prepared a transformant AO-*asolSC* with the plasmid (pUARA2-*asolSC*) by using the *A. oryzae* expression system. The metabolite profiles showed that AO-*asolSC* produced a new metabolite **2** (123 mg/kg of rice) (Figure 2). HR-MS data revealed the molecular formula to be $C_{24}H_{29}NO_4$ (unsaturation degree, 11) and the characteristic absorption at 283 nm in the UV spectrum suggested the presence of a 3-acyltetramate moiety derived from the tyrosine (Figure S3).[11] This finding was further supported by ^{1}H- and ^{13}C-NMR, and HSQC data of **2** (*p*-hydroxyphenyl group: δ_C 155.1, 130.3, 127.8, 115.7; tricarbonyl methane moiety of 3-acyltetramate: δ_C 194.0, 192.0, 175.3 and 101.9). Extensive analysis of the COSY correlations revealed that **2** possessed a decalin moiety. Detailed analysis of the NMR data including NOESY data (Figure S4) enabled us to determine the structure of **2** (except C5-stereochemistry), which is named as protodidymellamide α, as shown in Figure 1.

In the biosynthesis of tenellin, TenA, a chytochrome P450, catalyzed the characteristic oxidative ring expansion from tetramate to 2-pyridone.[12] To characterize the function of the homologous gene *asolA* (60% identity), a triple transformant AO-*asolSCA* was then prepared. This transformant produced two new metabolites **1** and **3** (**1**: 22 mg/kg, **3**: 84 mg/kg), and the UV spectra were closely related to the structurally related 2-pyridone polyketide metabolites (Figure 2, Figures S3 and S4).[13] The molecular formula ($C_{24}H_{27}NO_4$) was deduced from HR-MS and comparison of the NMR spectrum of **1** with that of natural product confirmed that **1** was identical with the antifungal metabolite didymellamide B isolated from a marine-derived fungus, *Stagonosporopsis cucurbitacearum* (previous name: *Didymella bryoniae*).[9]

In ^{1}H- and ^{13}C-NMR spectra of the second metabolite **3**, a part of signals appeared as a duplicated pair, suggesting that **3**

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was a 1:1 mixture of diastereomers. A comparison of ^{1}H- and ^{13}C-NMR data of **3** with those of **1** revealed that **3** possessed the same 3-acyl-2-pyridone scaffold (δ_H; 7.48; δ_C 188.1, 170.6, 158.8, 139.4, 111.5, 106.8), and that difference was located at a decalin moiety of **1**. Further detailed NMR analysis showed the presence of two partial structures, a conjugated diene connected with terminal methyl group and a β-hydroxycarbonyl moiety (Table S1, Figure S5), suggesting that **3** had a linear chain instead of trans-decalin via [4+2] cycloaddition as shown in Scheme 1. A treatment of **3** with acetic anhydride in the presence of DMAP at 85°C followed by methanolysis afforded **1** which was identical to the natural didymellamide B[9] in all respects. The conversion of **1** from **3** indicated that under the reaction conditions, C9-acetylation of **3** and the subsequent elimination took place to give triene **5a**, which underwent stereoselective cycloaddition to furnish acetate of **1** as a single isomer. This chemical transformation provided the further support on the structure of **3**.

These experimental results enabled us to discuss the biosynthetic pathway of didymellamide as shown in Scheme 1. In biosynthesis of tenellin, TenS, close homolog of PKS-NRPS AsolS, in collaboration with TenC generates a linear tetramate intermediate and the subsequent P450 TenA catalyzes a ring expansion to afford a linear 2-pyridone.[12] Together with [4+2] cycloaddition by PKS-NRPS hybrid LNKS,[14] we initially speculated that AsolS/AsolC catalyzed formation of a putative linear tetramate **4** and the subsequent cycloaddition gave **2** in the AO-*asolSC*. However, the conversion of **2** into **1** (route B) was not observed in the incubation of the single gene transformant AO-*asolA* (Figure S6). Isolation of β-hydroxyketone **3** is another key issue in the discussion of the didymellamide biosynthesis. Isolation of **3** as a 1:1 epimeric mixture suggested that **3** was not derived by the AsolS catalyzed reaction because ketoreduction of PKS was known to proceed in a highly stereoselective manner[15] and β-hydroxyketone **3** was obtained only as a tetramate form but not as a 2-pyridone form. Recently, we reported several unusual side reactions caused by the expression host *A. oryzae*.[16] Co-production of **3** with **1** suggested that the unusual product may be attributed to the action of the expression host *A. oryzae* hydratase which trapped linear intermediate **5a** to convert into **3** although nonenzymatic hydration can not be excluded. Considering all data shown here, we propose that the biosynthesis of **1** proceeds via route A (**4**-**5a**-**1**) and the [4+2] cycloaddition of **5a** proceed nonenzymatically. Non-enzymatic cycloaddtions were reported during the studies of the bifunctional DAases Sol5 (oxidase)[17] and LNKS (PKS)[14] as side reactions in which *endo*-adducts were preferentially obtained in the aqueous medium. In this case, the *endo*-selectivities observed in the aqueous medium were explained by a hydrophobic effect.

Recently, in the biosynthesis of decalin tetramate equisetin and Sch210972, the involvement of DAases, Fsa2[18] and CghA,[19] has been reported. In these cases, the wild type strains produced *endo*-adduct but the deletion mutant afforded a diastereomeric mixture of *endo*-/*exo*-adducts. While the cryptic gene clusters of putative tetramate polyketides, including pyrrolocin,[20] possess a DAase homolog, the *asol* and its homologous cluster for an anti-fungal agent apiosporamide from *Apiospora montagnei* NRRL25634,[13] do not have a DAase gene (Figure S2). This agreed that the non-enzymatic *endo*-selective cycloaddition of triene **5a** afforded trans-decalin **1** in the didymellamide biosynthesis. However, cis-decalin fischerin,[21] another congener of **1**, isolated from *Neosartoya fischeri* CBM-FA0156 may require the Diels-Alderase because the essentially same substrate triene **5b** is involved in the fischerin biosynthesis. Structural diversity of the tyrosine moiety might be derived by a series of oxidative transformations. These modification reactions are under investigation.

Conclusions

In summary, we have succeeded in the heterologous expression of the cryptic gene cluster found in *A. solani* to obtain a marine-derived antifungal agent didymellamide B from the *A. oryzae* transformant introducing PKS-NRPS, trans-ER and P450 genes *asolSCA*. The co-production of didymellamide shunt products enabled us to propose the exact sequence of oxidative ring expansion and non-enzymatic cycloaddition. It should be pointed out that **1** was not produced at a detectable level in the *A. solani*, even though the expression of the relevant genes was confirmed by RT-PCR of the host mRNA. Temporary or low expression of the gene clusters was often observed in the infection process of the phytopathogenic fungi.[1b] The successful production of didymellamide B in high yield proves that the heterologous expression system of *A oryzae* is a powerful tool to address the structural determination of a metabolite from a cryptic gene cluster.

Acknowledgments

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References and notes

1. (a) O'Connell, R. J.; Thon, M. R.; Hacquard, S.; Amyotte, S. G.; Kleemann, J.; Torres, M. F.; Damm, U.; Buiate, E. A.; Epstein, L.; Alkan, N.; Altmüller, J.; Alvarado-Balderrama, L.; Bauser, C. A.; Becker, C.; Birren, B. W.; Chen, Z.; Choi, J.; Crouch, J. A.; Duvick, J. P.; Farman, M. A.; Gan, P.; Heiman, D.; Henrissat, B.; Howard, R. J.; Kabbage, M.; Koch, C.; Kracher, B.; Kubo, Y.; Law, A. D.; Lebrun, M. H.; Lee, Y. H.; Miyara, I.; Moore, N.; Neumann, U.; Nordström, K.; Panaccione, D. G.; Panstruga, R.; Place, M.; Proctor, R. H.; Prusky, D.; Rech, G.; Reinhardt, R.; Rollins, J. A.; Rounsley, S.; Schardl, C. L.; Schwartz, D. C.; Shenoy, N.; Shirasu, K.; Sikhakolli, U. R.; Stüber, K.; Sukno, S. A.; Sweigard, J. A.; Takano, Y.; Takahara, H.; Trail, F.; van der Does, H. C.; Voll, L. M.; Will, I.; Young, S.; Zeng, Q.; Zhang, J.; Zhou, S.; Dickman, M. B.; Schulze-Lefert, P.; Ver Loren van Themaat, E.; Ma, L. J.; Vaillancourt, L. J. *Nat. Genet* **2012**, *44*, 1060; (b) Amselem, J.; Cuomo, C. A.; van Kan, J. A.; Viaud, M.; Benito, E. P.; Couloux, A.; Coutinho, P. M.; de Vries, R. P.; Dyer, P. S.; Fillinger, S.; Fournier, E.; Gout, L.; Hahn, M.; Kohn, L.; Lapalu, N.; Plummer, K. M.; Pradier, J. M.; Quévillon, E.; Sharon, A.; Simon, A.; ten Have, A.; Tudzynski, B.; Tudzynski, P.; Wincker, P.; Andrew, M.; Anthouard, V.; Beever, R. E.; Beffa, R.; Benoit, I.; Bouzid, O.; Brault, B.; Chen, Z.; Choquer, M.; Collémare, J.; Cotton, P.; Danchin, E. G.; Da Silva, C.; Gautier, A.; Giraud, C.; Giraud, T.; Gonzalez, C.; Grossetete, S.; Güldener, U.; Henrissat, B.; Howlett, B. J.; Kodira, C.; Kretschmer, M.; Lappartient, A.; Leroch, M.; Levis, C.; Mauceli, E.; Neuvéglise, C.; Oeser, B.; Pearson, M.; Poulain, J.; Poussereau, N.; Quesneville, H.; Rascle, C.; Schumacher, J.; Ségurens, B.; Sexton, A.; Silva, E.; Sirven, C.; Soanes, D. M.; Talbot, N. J.; Templeton, M.; Yandava, C.; Yarden, O.; Zeng, Q.; Rollins, J. A.; Lebrun, M. H.; Dickman, M. *PLoS Genet.* **2011**, *7*, e1002230.
2. (a) Fujii, R.; Minami, A.; Tsukagoshi, T.; Sato, N.; Sahara, T.; Ohgiya, S.; Gomi, K.; Oikawa, H. *Biosci., Biotechnol., Biochem.* **2011**, *75*, 1813–1817; (b) Tagami, K.; Liu, C.; Minami, A.; Noike, M.; Isaka, T.; Fueki, S.; Shichijo, Y.; Toshima, H.; Gomi, T.; Dairi, T.; Oikawa, H. *J. Am. Chem. Soc.* **2013**, *135*, 1260-1263; (c) Liu, C.; Tagami, K.; Minami, A.; Matsumoto, T.; Frisvad, J. C.; Ishikawa, J.; Suzuki, H.; Gomi, K.; Oikawa, H. *Angew. Chem. Int. Ed.* **2015**, *54*, 5748-5752; (d) Ye, Y.; Minami, A.; Mandi, A.; Liu, C. W.; Taniguchi, T.; Kuzuyama, T.; Monde, K.; Gomi, K.; Oikawa, H. *J. Am. Chem. Soc.* **2015**, *137*, 11846-11853
3. (a) Chooi, Y. -H.; Tang, Y. *J. Org. Chem.*, **2012**, *77*, 9933-9953; (b) Boettger, D.; Hertweck, C. *ChemBioChem* **2013**, *14*, 28 – 42.

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4. (a) Ichihara A.; Oikawa, H. *Biosci. Biotech. Biochem*. **1997**, *61*, 12-18; (b) N. Montemurre and A. Visconti, In Alternaria Biology, Plant Diseases and Metabolites. Vol.3, ed. by J. Chelkowski and A. Visconti. Elsevier. Amsterdam, 1992, pp. 449-557.
5. Kasahara, K.; Miyamoto, T.; Fujimoto, T.; Oguri, H.; Tokiwano, T.; Oikawa, H,; Ebizuka, Y.; Fujii, I. *ChemBioChem* **2010**, *11*, 1245-1252.
6. (a) Fujii, I.; Yoshida, N.; Shimomaki, S.; Oikawa, H.; Ebizuka, Y. *Chem. Biol.*, **2005**, *12*, 1301-1309; (b) Kasahara, K.; .Fujii, I.; Oikawa, H,; Ebizuka, Y. *ChemBioChem*, **2006**, *7*, 920-924.
7. Brian, P. W.; Curtis, P. J.; Hemming, H. G.; Unwin, C. H.; Wright, M. Nature, 1949, 164, 534.
8. Tabuchi, H.; Oikawa, H.; Ichihara, A. J. Chem. Soc,. Perkin Trans. 1 **1994**, 2833-2839.
9. Haga, A.; Tamoto, H.; Ishino, M.; Kimura, E.; Sugita, T.; Kinoshita, K.; Takahashi, K.; Shiro, M.; Koyama, K. *J. Nat. Prod.* **2013**, *76*, 750-754.
10. Eley, K. L.; Halo, L. M.; Song, Z.; Powles, H.; Cox, R. J.; Bailey, A. M.; Lazarus, C. M.; Simpson, T. J. *ChemBioChem* **2007**, *8*, 289–297.
11. Xu, W.; Cai, X.; Jung, M. E.; Tang, Y. *J. Am. Chem. Soc.* **2010**, *132*, 13604-13607.
12. (a) Heneghan, M. N.; Yakasai, A. A.; Halo, L. M.; Song, Z.; Bailey, A. M.; Simpson, T. J.; Cox, R. J.; Lazarus, C. M. *ChemBioChem* **2010**, *11*, 1508–1512; (b) Halo, L. M.; Heneghan, M. N.; Yakasai, A. A.; Song, Z.; Williams, K.; Bailey, A. M.; Cox, R. J.; Lazarus, C. M.; Simpson, T. J. *J. Am. Chem. Soc.* **2008**, *130*, 17988–17996..
13. Alfatafta, A. A.; Gloer, J. B.; Scott, J. A.; Malloch, D. *J. Nat. Prod*. **1994**, *57*, 1696.
14. (a) Auclair, K.; Sutherland, A.; Kennedy, J.; Witter, D. J.; Van den Heever, J. P.; Hutchinson, C. R.; Vederas, J. C. *J. Am. Chem. Soc.* **2000**, *122*, 11519-11520; (b) Witter, D. J.; Vederas, J. C. *J. Org. Chem.* **1996**, *61*, 2613-2623.
15. (a) Chooi, Y. H.; Tang, Y. *J. Org. Chem*. **2012**, *77*, 9933-9953; (b) Cox, R. J. *Org. Biomol. Chem.* **2007**, *5*, 2010.
16. R. Fujii, T. Ugai, H. Ichinose, M. Hatakeyama, T. Kosaki, K. Gomi, I. Fujii, A. Minami, and H. Oikawa, *Biosci. Biotechnol. Biochem.*, 2016, 80, 426-431.
17. (a) Oikawa, H.; Kobayashi, T.; Katayama, K.; Suzuki, Y.; Akitami, I. *J. Org. Chem*. **1998**, 63, 8748–8756; (b) Oikawa, H.; Suzuki, Y.; Naya, A.; Katayama, K.; Ichihara, A. *J. Am. Chem. Soc.* **1994**, *116*, 3605-3606.
18. Kato, N.;Nogawa, T.;Hirota, H.;Jang, J. H.;Takahashi, S.;Ahn, J. S.;Osada, H. *Biochem. Bioph. Res. Commun*. **2015**, *460*, 210.
19. a) Kakule, T. B.;Zhang, S. W.;Zhan, J. X.;Schmidt, E. W. *Org Lett*. **2015**, *17*, 2295; b) Sato, M.;Yagishita, F.;Mino, T.;Uchiyama, N.;Patel, A.;Chooi, Y. H.;Goda, Y.;Xu, W.;Noguchi, H.;Yamamoto, T.;Hotta, K.;Houk, K. N.;Tang, Y.;Watanabe, K. *ChemBioChem*. **2015**, *16*, 2294.
20. Kakule, T.B.; Jadulco, R.C.; Koch, M.; Janso, J.E.; Barrows, L.R.; Schmidt, E.W. *ACS Synth. Biol*. **2015**, *4*, 625−633
21. Fujimoto, H.; Ikeda, M.; Yamamoto, K.; Yamazaki, M. *J. Nat. Prod.* **1993**, *56*, 1268.

Supplementary Material

Supplementary data (detailed experimental procedures, LC-MS data, and NMR data) associated with this article can be found in the online version at xxx.

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HIGHLIGHT

- Heterologus production of fungal metabolite with 3 genes
- biosynthetic gene dependent production of fungal polyketide
- non-enzymatic [4+2] cycloaddition of highly reactive linear triene
- endo-selective Diels-Alder reaction generating trans-decalin with 2-pyridone

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